CLINICAL MICROBIOLOGY REVIEWS, Oct. 1993, p. 367–381
0893-8512/93/040367-15$02.00/0

Vol. 6, No. 4

Antifungal Susceptibility Testing

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INTRODUCTION

The frequency of serious fungal infections is rising, and this trend has been attributed to such factors as the increasing use of cytotoxic and immunosuppressive drugs to treat both malignant and nonmalignant diseases, the increasing prevalence of infection due to human immunodeficiency virus type 1, and the widespread use of newer and more powerful antibacterial agents (5, 7). Fortunately, this increase in fungal infections has been accompanied by the development of new, less toxic, systemically active alternatives to amphotericin B such as fluconazole, itraconazole, and the various amphotericin B lipid formulations (129). With this proliferation of antifungal agents, therapeutic options are more numerous, and the clinician must now choose among them. This decision is made more difficult by the steady stream of reports of putative drug resistance to one or more antifungal agents (6, 13, 16, 26, 32, 33, 38, 39, 46, 47, 62, 69, 70, 72, 75, 89, 93, 103, 105, 119, 120, 126, 136, 138, 146, 150–152). As these reports are not always convincing or well controlled, making an informed antifungal therapy choice is not easy.

As with bacterial infections, the clinician would like to be guided by knowledge of local epidemiological patterns derived from drug susceptibility testing results. Unlike antibacterial susceptibility testing, however, reliable antifungal susceptibility testing is still largely in its infancy. A prodigious array of techniques has been described, but without standardization, the various methods have produced widely discrepant results because of substantial dependence on such factors as pH, inoculum size, liquid versus solid media, medium formulation, time of incubation, and temperature of incubation. Even when a group of laboratories agrees upon a general technique, widely discrepant results may be obtained for some antifungal agents if all procedural details are not precisely defined (19). Finally, once a technique is selected and the MIC for an isolate is determined, there are few useful data available to guide MIC interpretation. Many authors have sought in vivo-in vitro correlations in animal models or with isolates from patients: some have found a correlation (3, 14, 39, 72, 85, 93, 104, 105, 119, 120, 122, 127, 128, 136, 139, 149), but others have not (20, 47, 55, 73, 86, 127, 134). This situation is obviously unsatisfactory, and a substantial amount of work has been undertaken to resolve these difficulties. While a complete solution is not available, considerable progress has been made, and it is the purpose of this review to bring this information into perspective.

EARLY STUDIES

Antifungal susceptibility testing was not relevant until the introduction in the 1950s of amphotericin B. That this was nearly 30 years after the discovery of the first antibacterial agents explains in part the immaturity of antifungal susceptibility testing today. Even then, antifungal susceptibility testing lay fallow for many years because, while not all fungal infections responded to amphotericin B, there were no alternatives. It was only with the development of 5-fluorocytosine and, more recently, the azole antifungal agents

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that differences within and between species started to become apparent. Even so, only a small number of laboratories routinely performed antifungal susceptibility testing. In 1986, Calhoun et al. (19) surveyed 350 hospital laboratories. Only 41 of the 210 respondents were performing antifungal susceptibility tests, with 45% of these laboratories testing only one to five isolates during the previous year. A variety of methods was being used: most commonly, a broth technique that had been derived from an antibacterial susceptibility testing method. When seven laboratories used this common, published methodology to test five *Candida albicans* isolates against amphotericin B and 5-fluorocytosine, results that varied by as much as 512-fold were obtained. Of significant interest, however, was the observation that while the absolute values of the MICs for each isolate varied greatly among laboratories, the relative susceptibilities of the isolates varied little among laboratories. In other words, the rank order of the isolates was quite constant despite wide variability in endpoint results. In a follow-up study conducted by three laboratories (51), another set of *Candida* isolates was tested against amphotericin B, 5-fluorocytosine, and ketoconazole. In this study, however, no methodology was prescribed. Rather, each group used the method with which they were most familiar, and the implemented techniques included broth dilution, agar dilution, and turbidimetric techniques. Again, while the actual MICs varied by as much as 50,000-fold, the rank order of the isolates produced by the results from each laboratory varied only slightly.

Given these data and the growing interest in antifungal susceptibility testing, it was clear that standard, reproducible techniques for antifungal susceptibility testing were needed. Many approaches to antifungal susceptibility testing have been reported, but not all readily lend themselves to standardization. Tests that measure the rate of elongation of germ tubes (75, 136, 151) are tedious to perform and are useful only for *C. albicans*, the one species in the genus that forms germ tubes within 3 h in serum. An automated system based on the dynamic growth of a single hypha as measured by microscope, camera, television monitor, video tape recorder, and microcomputer was recently described (102, 154), but it appears impractical for routine use. Examination of even more subtle morphologic effects is possible and would be applicable to a wider range of fungi (121, 136), but such approaches are highly subjective. Measurements of uptake of radiolabeled metabolites (30, 31, 35, 56) or reduction of colored substrates (79, 124) have been used to assess phagocyte-mediated damage to *C. albicans*, *Aspergillus fumigatus*, *Rhizopus arrhizus* (=*R. oryzae*), and *Coccidioides immitis* and could readily be adapted to measuring the effect of antifungal drugs on fungi (88, 140, 143). However, these tests are indirect since lack of metabolic uptake of the given substrate does not necessarily imply death. Moreover, they require special material-handling procedures in the case of the use of radiolabeled substrates and relatively large inocula in order to produce a measurable amount of product in the case of colored substrates. Approaches that use flow cytometry (60, 116–118) or viable colony counts (71, 95) have been described but are applicable only to organisms that disperse freely and are technically or physically demanding. Measurement of biomass by bioluminescence spectrophotometry has been described (95). This technique used measurements at multiple points along a dose-response curve to allow computation of a relative inhibition factor (100), and although the method is intriguing, instrumentation that would make this approach practical has not been developed further.

Agar-based techniques have been used extensively by a few laboratories because they are simple, economical, and easy to perform simultaneously on large numbers of organisms. Either the fungus can be placed in the agar and the antifungal agent can be placed on the surface of the agar (disk or well diffusion) or vice versa (agar dilution). These techniques suffer from substantial dependence on inoculum, temperature, and duration of incubation (15, 66, 67, 75, 94). The estimation of the size of inhibitory zones can be difficult because of partial growth inhibition (37, 67, 139) or the presence of "persistor" [sic] colonies within otherwise clear zones of inhibition (145). Finally, the absolute MICs of the azoles tend to be lower than those produced by broth assays (15, 94). Some of these problems might be overcome by careful standardization (59), but the physical-chemical properties of the antifungal agents to be tested and their interaction with the agar cannot be altered. Agar is not a chemically defined material, and, as has been noted in assays of the aminocyclitol antibiotics, agars from different sources can produce different results because of variations in such simple properties as cation concentration (66). Such variations are also potentially relevant to antifungal assays (94). In addition, some (61, 68), but not all (130), authors have found that amphotericin B and the azoles tend to deteriorate when stored in dilute form or dried on antibiotic assay disks. Some of the azoles, especially miconazole, ketoconazole, and itraconazole, are relatively insoluble and may diffuse poorly in an agar diffusion system (61, 121, 129). Finally, some species of *Candida* other than *C. albicans* are inhibited when grown in agar (97). Despite these problems, it has been possible to develop routine testing methods that use an agar diffusion format (133). Reproducible results that correlate well with broth dilution MICs have been obtained (37, 115, 130), and standard techniques for performing this assay have been described (111, 137). In addition, many reports of amphotericin B resistance have used agar dilution methods (32, 33, 62, 87, 89, 103). Whether currently proposed broth methods can detect this resistance needs to be determined (see reference 50 and below).

Broth dilution methods for antifungal susceptibility testing were the most commonly used techniques in the United States in the previously mentioned survey (19). Because of this and the other factors just discussed, broth dilution methods have been the focus of the most recent efforts at test standardization. A number of collaborative studies of broth dilution methods have now been carried out both independently (43, 63, 111, 135, 144) and under the aegis of the Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards (NCCLS) (19, 44, 48, 51, 107, 108). As a result, many of the factors that influence (often profoundly) this technique have been identified, and a standard method that minimizes their adverse effects has been proposed (92). This method has been proposed exclusively for testing *Candida* spp. and *Cryptococcus neoformans*; reproducible methods for other organisms are still under development (see below). In the next section, we review the variables involved in implementing a broth-based susceptibility test, with special emphasis on how each variable affects the proposed NCCLS reference method (Table 1). Knowledge of these variables is helpful in understanding the relative importance of the various details of the assay and in interpreting studies that use variant methodologies.

FIG. 1. Trailing endpoints. A *C. albicans* isolate was tested for susceptibility by using the proposed NCCLS reference method, and percent transmission at 530 nm was determined. The lines marked 80 and 90% are the percent transmission of 1:5 and 1:10 dilutions of the drug-free control tube, respectively. The amphotericin B curve (□) makes a very rapid transition from marked turbidity to 100% transmission, while the fluconazole curve (×) trails markedly over this range. The amphotericin B MIC for this isolate is 0.5 μg/ml (first tube with 100% transmission), and the fluconazole MIC is 0.25 μg/ml (first tube at or above 80% transmission).

FACTORS THAT INFLUENCE ANTIFUNGAL SUSCEPTIBILITY TESTING

Endpoint Definition

Endpoint determination is probably the most significant source of interlaboratory variability for the azole antifungal agents and, under some conditions, 5-fluorocytosine. For these agents, inhibition does not develop abruptly over a small concentration range. Rather, after an initial prominent drug effect, small amounts of turbidity may persist for many, if not all, drug concentrations tested (Fig. 1). As a result, laboratories that insist on stringent reduction of turbidity report much higher MICs than do laboratories that adopt endpoints that are tolerant of small amounts of turbidity.

In contrast, endpoints with amphotericin B and cilofungin do not show this phenomenon, and comparison of the kinetics of the onset of drug effect has provided some explanation for differences between these drugs (54, 85). At or above some critical amphotericin B concentration, turbidimetric measurements demonstrate that growth ceases soon after exposure to the drug. However, growth does not begin to slow until approximately one doubling time after exposure to 5-fluorocytosine or the azole antifungal agents, and growth is not fully arrested until some time later. These observations demonstrate that this trailing endpoint is the result of growth occurring for a period of time prior to the onset of complete drug effect. Predictably, the trailing endpoint problem has been shown to worsen with increasing initial inoculum (127), and this may be the reason that recent collaborative trials have found better reproducibility with a smaller inoculum (27, 44, 48). It has been suggested that this problem could be reduced by the addition to the medium of a protein synthesis inhibitor such as doxycycline (96, 101), but this approach has not been exploited further.

Given these observations, several approaches to endpoint definition are possible. One is to describe the endpoint with carefully selected phrases. This was done in two recent collaborative studies in which endpoint determinations of "optically clear," "slightly hazy," and "prominent decrease in turbidity from control" were used (44, 48). (These descriptions are also referred to as 0, 1+, and 2+, respectively.) It was found that the endpoints that allowed some

TABLE 1. Variables that influence antifungal susceptibility testing[a]

Drug	Trailing endpoint present?	MIC rises with larger inoculum	Antagonists in medium	Effect of decreasing pH	Effect of temp
Polyenes	No: amphotericin B (54)	No: nystatin (15), amphotericin B (54, 57)	Sterols (78)	Raises amphotericin B MIC (9, 40)	Amphotericin B MICs at 22°C both higher and lower than at 34°C (9)
5-Fluorocytosine	Under some conditions (54), but not others (12)	Yes (12, 15, 54, 57)	Purines and pyrimidines (66, 113, 114)	Lowers MIC (40)	MIC lower at 25°C than at 37°C (99); higher for some isolates at 32°C than at 37°C (12)
Azoles	Yes: miconazole (54), ketoconazole (48), fluconazole (44, 48)	Yes: miconazole (54, 75), ketoconazole (75), tioconazole (75), SCH39304 (85), fluconazole (57), others (94)	Postulated for clotrimazole (66) and several other azoles (95)	Raises MIC: fluconazole (85, 127), SCH39304 (85), ketoconazole (40, 90, 127), miconazole (40)	MIC of terconazole lower at 25°C than at 37°C (99)
Cilofungin	No (85)			Raises MIC (86)	

[a] Not shown are the effects of time: MICs of amphotericin B, nystatin, 5-fluorocytosine, ketoconazole, miconazole, and several other azoles have been shown to rise with longer incubation (11, 12, 15, 36, 75, 94). For further reviews of the effects of these variables, see references 49, 52, and 91. Numbers in parentheses are references.

turbidity gave more reproducible results and also produced better agreement with known patterns of in vivo resistance. While some trade-offs were involved, the "prominent decrease in turbidity from control" endpoint was the most consistently reproducible.

Communicating the precise meaning of "prominent decrease in turbidity from control" is, however, problematic. An approach to the resolution of this problem has been described by Espinel-Ingroff et al. (44). These authors demonstrated that the simple method of diluting the drug-free control 80% after incubation (8 parts medium, 2 parts yeast suspension) provided a very good approximation of "prominent decrease in turbidity." To illustrate this relationship, Fig. 1 shows the turbidity of a strain of *C. albicans* after incubation with different concentrations of fluconazole and amphotericin B. As can be seen, an 80% dilution is easily distinguished from the residual turbidity and for this reason is likely to result in agreement when read visually by different observers. On the other hand, the turbidity produced with a 90% dilution of the drug-free control provides a less satisfactory endpoint definition since it is very near the residual trailing endpoint. Similar trailing endpoints were observed with the sulfonamides in antibacterial testing and a similar solution was applied (8), thus lending precedent to this approach.

Another approach has used spectrophotometric determinations to quantitate turbidity (4, 9, 11, 53, 54). Similar in spirit to visual estimations of relative turbidity, spectrophotometric measurements lend themselves better to numeric manipulation as well as holding the potential for eliminating subjective interpretations. In these approaches, the turbidity of the drug-free controls is defined as 100%, and the turbidities of the test dilutions are scaled to this value. The concentration at which the turbidity is reduced to a specified percentage, typically 50 or 30% of the drug-free control, is then taken as the endpoint and called the 50 or 30% inhibitory concentration (IC_{50} or IC_{30}, respectively). Several studies have found that such an approach eliminates inoculum dependence (4, 9, 53, 54), but this method requires specific instrumentation to perform the measurement.

Endpoint determinations that examine killing have been described. Such studies can be implemented as kinetic turbidimetric and time-kill curves or as determinations of minimum lethal concentrations (MLCs) (147, 149). A few reports have suggested that MLCs may be more important than MICs in predicting in vivo resistance (24, 83, 149). This general area has not yet been pursued in detail. If the experience with bacteria is any guide (142), defining a reproducible technique for MLCs will likely require more work than has already been required to define a technique for MICs.

Inoculum Size

Starting concentrations for yeasts in broth assays have ranged from 10^2 to 10^6 per ml. Unless a relative turbidimetric endpoint is used, MICs for most drugs increase with increasing inoculum over this range, regardless of test format, sometimes as much as 512-fold (12, 15, 53, 57, 75, 85, 94). Both intra- and interlaboratory reproducibilities tend to improve with smaller inocula (27, 44, 48). Recent collaborative efforts found that an inoculum in the range of 0.5×10^3 to 2.5×10^3 per ml produced the most interlaboratory agreement, and this value has been adopted in the proposed NCCLS reference method (92).

Inoculum Preparation

In a recent collaborative study of inoculum preparation, four techniques were compared by three laboratories: a spectrophotometric method, the Wickerham card method, a hemacytometer method, and the Prompt inoculation system (107). Yeast suspensions were adjusted in an agreed upon fashion, using each system in order to produce an inoculum of 1×10^6 to 5×10^6 per ml, and then plated to assay for viable CFUs. The Wickerham card method and the Prompt inoculation system were the most variable and often failed to produce the desired inoculum. Adjustment by matching the turbidity at 530 nm of a 0.5 McFarland standard was the best method; this was followed closely by hemacytometer counts. The hemacytometer method displayed greater variability, apparently because of consistently aberrant results from one laboratory. On the basis of these results, the proposed NCCLS reference method has adopted spectrophotometric standardization of the inoculum. While such techniques as staining with a fluorescent dye (25) and the use of a Coulter counter (41) have been suggested, these techniques require equipment that may not be readily available.

Incubation Time and Temperature

Although the magnitude of the effect varies from drug to drug, MICs tend to increase with increasing incubation periods (11, 12, 15, 36, 75, 94). For example, Brass et al. found that the ketoconazole MIC for a strain of *C. albicans* increased 10-fold in a broth method and 2,000-fold in an agar dilution assay during 7 days of observation, while the nystatin MICs rose by, at most, one dilution (15). MICs for yeasts are usually stable by 4 days (94). Temperature changes are also significant. The MIC has been described to both rise and fall with temperature changes from 22 to 37°C (9, 12, 99). The standard temperature for antibacterial susceptibility testing in the United States is 35°C, and this appears to be a good choice because it generally supports fungal growth better than 30°C (108) and because MIC results are less variable than at 37°C. In one surprising study, macro- and microbroth procedures for fluconazole and the investigational triazole SCH39304 in RPMI 1640 were compared at two inocula (10^2 and 10^4 CFU/ml) and two temperatures (35 and 37°C) (27). While the various combinations of temperature and inoculum gave nearly identical results at 35°C, there was as much as an eightfold difference in results at 37°C. Finally, a collaborative study of 100 isolates in 13 laboratories found that, for tests of amphotericin B, ketoconazole, and 5-fluorocytosine in RPMI 1640 at 35°C, the most consistent overall results were obtained after incubation at 35°C for 48 h for *Candida* spp. or 72 h for *Cryptococcus neoformans* (48). In both cases, these times of reading correspond to the second day on which growth is readily apparent in the drug-free control tube.

On the basis of these results, the proposed NCCLS reference method specifies incubation at 35°C for 48 h for *Candida* spp. or 72 h for *Cryptococcus neoformans*.

Media

As with testing of bacteria, use of different media may give substantially different results (27, 36, 37, 57, 66, 108). When conventional, undefined media (e.g., Sabouraud agar) were critically compared with the totally synthetic medium SAAMF (synthetic amino acid medium, fungal), substantial differences in 5-fluorocytosine MICs were noted (66), and

these differences turned out to be due to antagonism of 5-fluorocytosine action by purines and pyrimidines in the media (66, 113, 114). Other factors may affect other antifungal agents: sterols in the medium can interfere with the effects of polyenes (78), and as yet undefined factors have been postulated to interfere with the effects of azoles in some media (67, 94). Even well-defined media can hold surprises: the use of morpholinepropanesulfonic acid (MOPS)-Tris to buffer SAAMF was found to inhibit the action of 5-fluorocytosine, independently of its effect on pH (18). This effect was later determined to be due entirely to the Tris (80). Differences that are not due to the presence of antagonistic substances may be due to pH. Not all media have the same native pH or a similar buffering capacity, and the rate of growth of the test organism may be affected by pH (18). Further, acidic conditions have been found to raise the MICs of amphotericin B (9, 40), fluconazole (85, 127), SCH39304 (85), ketoconazole (40, 90, 127), miconazole (40), and cilofungin (86) but to lower 5-fluorocytosine MICs (40). This effect on MICs can be substantial: ketoconazole MICs are 64- to 1,000-fold higher at pH 3.0 than at pH 7.4 (90, 127). Thus, it is clearly desirable to use a buffered medium. The optimum pH, however, is less clear. While the neutral pH of 7.0 is intuitively attractive, McIntyre and Galgiani (86) found that cilofungin MICs for *C. albicans* spanned a wide range at pH 7.0 (0.08 to 2.5 μg/ml) but a much narrower range at pH 3.0 (5 to 10 μg/ml). When isolates for which MICs were 0.08 and 1.25 μg/ml at pH 7.0, but identical (5.0 μg/ml) at pH 3.0, were tested in a rat model of disseminated candidiasis, the 50% effective doses of cilofungin were identical, thus suggesting that the pH 3.0 MIC results were more meaningful. While MICs for some isolates of three other species studied by these authors were >80 μg/ml at pH 7.0, these isolates were not tested in the animal model.

The insolubility of some compounds may be a final source of difficulty. When working from highly concentrated stock solutions, it is important that all dilutions be made in media that will maintain the drug in a soluble form. When working with bifonazole, an imidazole derivative, Plempel et al. demonstrated that drug concentrations could be artifactually lowered by as much as 10-fold if this principle was not observed (110). To address this problem, the proposed NCCLS reference method suggests that amphotericin B and ketoconazole be dissolved in dimethyl sulfoxide.

On the basis of these results, a completely synthetic, buffered medium would appear to be optimal. Even though it may be suboptimal for some drugs, pH 7.0 has been the most commonly used pH. Most current investigations, including the proposed NCCLS reference method, have adopted the synthetic cell culture medium RPMI 1640, buffered to pH 7.0 with MOPS. RPMI 1640 is readily available commercially and is relatively inexpensive. This medium and buffer are not sacrosanct: similar results with other buffers (80) and the synthetic HR (high-resolution) medium (108) can clearly be obtained.

Results Obtained with the Proposed NCCLS Reference Method

Fromtling et al. (48) and Espinel-Ingroff et al. (44) have recently reported results from the first major studies to use the NCCLS assay. Of note, these two studies present somewhat overlapping sets of data: in Fromtling's study, 35 strains of *C. albicans*, 15 strains of *C. tropicalis*, 15 strains of *C. parapsilosis*, 10 strains of *C. lusitaniae*, 10 strains of *Torulopsis glabrata*, and 15 strains of *Cryptococcus neoformans* were studied in 13 independent laboratories for susceptibility to amphotericin B, 5-fluorocytosine, and ketoconazole, while Espinel-Ingroff's study made use of the same isolates in 5 of the same 13 laboratories, but these 5 laboratories also tested fluconazole. Thus, Espinel-Ingroff's data for fluconazole are unique, but the other data from this study are a subset of the Fromtling study and will not be discussed further.

Many of the results from these studies have been alluded to during the previous discussion, but there are additional noteworthy points. First, the choice of an inoculum of 0.5×10^3 to 2.5×10^3 per ml, an endpoint of "prominent decrease in turbidity," and reading at 48 h for *Candida* spp. and 72 h for *Cryptococcus neoformans* constitute a compromise made to optimize interlaboratory agreement for all of the study drugs. A result was said to be in agreement if it was within 1 dilution of the modal result for that strain by all laboratories, and by these criteria, amphotericin B produced ≥85% interlaboratory agreement for a variety of starting conditions and endpoint definitions. Ketoconazole, on the other hand, required precisely the above conditions in order to achieve even 75% interlaboratory agreement. Second, the interlaboratory agreement produced with this technique is better than has been achieved in the past but is still not perfect. The agreement for amphotericin B is 90%; for 5-fluorocytosine, 85%; for fluconazole, 88%; and for ketoconazole, 75%. On the basis of these and other studies, it is clear that this rank order for reproducibility is a feature of the drugs and not the assay system. For example, when Shawar et al. (135) recently tested a variety of broth microdilution and semisolid agar dilution methods in two laboratories, they found that results for amphotericin B and 5-fluorocytosine were highly reproducible, fluconazole had good but not perfect reproducibility, and itraconazole and SCH39304 had poor reproducibility. Third, this method produces a broad range of MICs of 5-fluorocytosine (0.125 to 64 μg/ml), ketoconazole (0.03 to 2 μg/ml), and fluconazole (0.12 to 64 μg/ml), but not amphotericin B (0.25 to 1.0 μg/ml). These results were obtained despite inclusion in the study of a number of *C. lusitaniae* isolates, a species for which elevated MICs by agar dilution methods in cases of amphotericin B resistance have frequently been described (62, 87, 89, 103). These data suggest that either there were no resistant *C. lusitaniae* isolates in the test collection or the assay system is relatively insensitive to amphotericin B resistance.

This apparent inability to detect resistance to amphotericin B may in part be a function of the choice of a broth system rather than an agar system. While MICs by agar dilution can range over more than 2 orders of magnitude (33, 89), results by broth dilution methods have usually varied over narrower ranges. For example, in the relatively large series of amphotericin B-resistant *Candida* infections reported by Powderly et al. (120), MICs for the 29 described isolates were between 0.4 and 7.5 μg/ml by a macrobroth technique (yeast extract broth, 10^6 yeast cells per ml, 30°C, 48 h). Other authors have also described amphotericin B MICs by macrobroth techniques that varied over a similar range for both *Cryptococcus neoformans* (9) and *C. albicans* (19, 122). The use of RPMI 1640 in the proposed NCCLS method may have further narrowed the range of amphotericin B MICs produced by the proposed method. Ghannoum et al. (57) recently have described results of MIC testing for 21 isolates of *Cryptococcus neoformans*, using 10^4 yeast cells per ml and a 48-h incubation at 35°C in a microtiter format. Four media (MOPS-buffered pH 7.0 RPMI 1640,

MOPS-buffered pH 7.0 yeast nitrogen base [YNB], MOPS-buffered pH 7.0 SAAMF, and pH 5.4 YNB) were studied. The data demonstrate compression of the amphotericin B MIC range when RPMI 1640 is used, and these authors conclude that pH 7.0 MOPS-buffered YNB is preferable to RPMI 1640 for testing amphotericin B susceptibility of *Cryptococcus neoformans*. As a final demonstration of this phenomenon, when 234 blood isolates of *Candida* spp. were tested by the proposed NCCLS method, the fluconazole MICs ranged from 0.125 to >64 μg/ml, but all amphotericin B MICs were between 0.125 and 1 μg/ml (123).

Taken together, these observations suggest that, while broth systems can detect amphotericin B resistance, the proposed NCCLS method does not appear to be well suited to detect resistance to this particular drug. Two approaches to resolving this problem can be proposed. First, if the proposed NCCLS reference method produces a narrow range of values, more dilution steps within the expected range might be required to demonstrate a distribution of values and to consistently allow discrimination between isolates. This concept is supported by recent data presented by Dignani et al. (34). With a microplate adaptation of the proposed NCCLS method (4), four strains of *C. lusitaniae* with MICs ranging from 0.312 to 2.5 μg/ml were identified, and these MICs were found to correlate with the outcome of amphotericin B therapy in a mouse intravenous model of candidiasis. When these isolates were tested by one of us (J.H.R.) with the proposed NCCLS method (the testing was done in a blinded fashion during the testing of the just-described 234 blood isolates of *Candida* spp.), a MIC of 2 μg/ml on five occasions and a MIC of 1 μg/ml on two other occasions were found for the isolate with a microbroth MIC of 2.5 μg/ml. Thus, for this isolate, a modal MIC that was higher than that for any of the other tested isolates was found, but given the expected one-tube, between-run variation in MIC results, it is not reasonable to expect the proposed NCCLS method to consistently detect such a difference. Alternatively, if smaller concentration steps do not resolve this problem, some modification of the media or test conditions will need to be developed. This is an area of active investigation.

Despite these difficulties, the proposed NCCLS reference methodology should be useful as a touchstone during the next stages in the evolution of antifungal susceptibility testing. In addition to the points mentioned above, two other areas need to be addressed. First, the proposed NCCLS reference method is a macrobroth method that uses 1.0-ml volumes in test tubes. This technique is cumbersome and should be replaced by a microbroth method that gives identical results as soon as possible. Even though the difference is only a change in scale of the assay, MICs by microbroth methods have not been the same as those obtained with a macrobroth technique: microbroth MICs have been reported to be both higher (43, 44, 85) and lower (27) than macrobroth levels. In a recent study that compared the proposed NCCLS reference macrobroth method with a microbroth method that simply reduced the 1-ml macrobroth volumes to 0.2 ml (43), interlaboratory agreement was optimal and similar for the two techniques with the same inoculum, incubation, and endpoint conditions. However, the resulting microbroth MICs were higher than the macrobroth MICs. Of note, this study compared only results obtained by identical methods (e.g., microbroth and macrobroth with the same starting inoculum, time to reading, and reading endpoint), rather than seeking to define an independent microbroth method that produced the same results as the macrobroth method. Agitation of the microtiter plates prior to reading may be useful to help eliminate these discrepancies and to improve reproducibility (4, 57, 85).

Even more innovative simplifications could be developed. For example, the relative turbidity of the growth of an organism in a single concentration of a drug was used to classify organisms by comparison to the typical growth of the given species in drug-free media (98). While this technique does not give an actual MIC, it could, with carefully chosen drug concentrations, prove to be a simple way to distinguish resistant and susceptible strains.

Finally, achieving consensus on a reference procedure will make it possible to begin to assess the importance of in vitro drug resistance in therapeutic failures. As will be seen below, it is highly likely that a correlation based on isolate rank order will exist, but it is much less certain that the actual MIC generated by the test will have a meaningful or predictable relationship to achievable serum levels of that drug.

OTHER ORGANISMS

Testing of organisms other than *Candida* spp. and *Cryptococcus neoformans* presents a special set of problems (49). While *Candida* spp. may exhibit some hyphal forms, *Candida* spp. and *Cryptococcus neoformans* exist predominantly in the laboratory and during infection in a small, round yeast form. These yeast cells are easily counted, diluted, and transferred from one vessel to another, making a wide variety of test procedures possible. And, in part because the invasive form of the organism is easily studied in the laboratory, it seems likely that susceptibility testing would predict clinical response. It is for these reasons, as well as the fact that the majority of fungal infections are due to these organisms, that most published studies of antifungal susceptibility testing and all of the current efforts towards standardization have focused exclusively on studies of *Candida* spp. and *Cryptococcus neoformans*.

The dimorphic fungi which exist in nature as molds but which have yeast forms during infection (*Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Sporothrix schenckii*) can be maintained as yeasts in the laboratory. Special laboratory precautions are required, and the technique would need to be modified in order to accommodate the typically slower growth of these organisms (24, 45, 49, 64), but they could potentially be studied in the same fashion as *Candida* spp. and *Cryptococcus neoformans*. In making such adaptations, it will, however, be critical to pay heed to the individual properties of the various fungi. For example, some strains of *S. schenckii* are exquisitely sensitive to temperatures just above 35°C, and it would be critical that this temperature never be exceeded (132).

Filamentous fungi such as *Aspergillus* spp. present a different picture. These organisms do have a small, round, conidial form that is easily counted, diluted, and transferred, but the invasive form of the organism is a hyphal form that cannot be accurately counted, diluted, or transferred because of both the ready adherence of hyphae to surfaces and the macroscopic, interconnected nature of the hyphal mat. Because of these difficulties, most studies of molds published to date have started with an inoculum of conidia and terminated upon detection of the presence or absence of hyphal growth (29, 121). Lack of growth in such studies may be due to inhibition of germination rather than inhibition of hyphal growth, and it is less certain that such approaches would correlate with clinical response. Susceptibility testing

of aspergilli has been reviewed recently (29), and the issues involved in defining a reproducible procedure have only begun to be defined (42). As noted above, all of these studies suffer from potential problems related to starting the assay with an inoculum of conidia or asexual propagules rather than with one of hyphae.

Preliminary data obtained by Martin et al. suggest that the distinction between starting with conidia versus hyphae may be more than academic (82). In their study, germinating conidia were observed and harvested when discrete germ tubes, but not intertwined hyphae, were present. Such an inoculum should, in theory, exhibit behavior that more closely approximates that of mature hyphae. Similar amphotericin B MICs for *Aspergillus* spp., *Fusarium* spp., and the dematiaceous fungi were found with both resting and germinating conidia, while higher amphotericin B MICs for *R. arrhizus* were found with germinated sporangiospores. Itraconazole MICs were either the same or only slightly higher for the germinated forms. However, MLCs of itraconazole, but not amphotericin B, for the dematiaceous fungi and *Aspergillus* spp. were much higher in the germinated forms. Taken together, these data suggest that substantial work to define appropriate testing procedures for the molds remains to be done.

CORRELATION OF IN VITRO TESTING WITH IN VIVO OUTCOME

While it is obviously important to have a reproducible assay, an assay is needed only when clinical resistance exists and is predicted by the assay. Adequate studies to correlate in vitro test results with in vivo efficacy in humans have not yet been done. However, many independent pieces of information suggest that it will be possible to demonstrate a correlation. Three types of data are available. First, there are animal studies in which the in vitro-in vivo correlation has been directly addressed. Second, there are a substantial number of clinical observations in which clinical recurrence or failure during drug therapy has been correlated with reduced in vitro susceptibility. Finally, there are a small number of clinical trials during which isolates were collected and tested for susceptibility and for which the results were correlated with clinical outcome.

Animal Studies

The relationship between in vitro results and in vivo efficacy for the polyenes has been examined with a number of animal models (Table 2). The most exhaustive study is that of O'Day et al. (93). Microbroth MICs produced an excellent correlation for both amphotericin B and natamycin (another polyene), while microbroth MLCs and an agar diffusion assay were less predictive and tended to incorrectly classify isolates unless the tests were performed multiple times. Similar results were obtained in a mouse intravenous infection model (3). In the study by Fisher et al. (47), three *Candida* isolates with amphotericin B MICs of 0.2, 0.39, and 0.78 μg/ml were tested. In vivo, amphotericin B was active against all three isolates, but the reduction in kidney and liver CFU per gram was least for the isolate with the highest MIC. This result, however, is difficult to interpret because of other differences between the isolates: different inocula were required to produce infection, and the organ CFU per gram in the untreated controls varied over a 3-log range.

All four published studies of in vivo-in vitro correlation for 5-fluorocytosine have demonstrated a correlation (3, 34, 122, 139). The most thorough study is that of Stiller et al. (139), in which 40 isolates with varying in vitro susceptibilities were examined. This study demonstrated a clear-cut statistical correlation, but unfortunately there was substantial overlap between the amount of 5-fluorocytosine required to control infection caused by isolates with low 5-fluorocytosine MICs versus those with high MICs. Thus, despite the presence of an obvious in vivo-in vitro correlation, no clear breakpoint to distinguish 5-fluorocytosine-susceptible and -resistant isolates could be established (Fig. 2). These authors conclude that their isolates did not contain a distinct subset of resistant isolates but rather were composed of a group of subisolates with a continuous spectrum of susceptibilities. The other three studies of 5-fluorocytosine are either too small or as yet incompletely published and do not shed more light on this problem.

The largest number of studies are those of the azole antifungals. While these studies have usually demonstrated an in vivo-in vitro correlation, they also provide several useful lessons. First, as with the above-mentioned study by O'Day et al. (93), the degree of correlation depends strongly on the particular in vitro test. Ryley et al. (128) examined two isolates that were clearly resistant to ketoconazole in vivo in both humans and three different animal models. The isolates were found to be resistant by two broth methods and two agar diffusion methods, but by an agar dilution method the MICs for the isolates were the same as those for the control isolates. Polak et al. (112) studied the ketoconazole susceptibility of 58 *C. albicans* isolates, using 16 different agar and broth methods, and correlated these results with the dose of ketoconazole required to keep 50% of the animals alive (50% effective dose [ED_{50}]). Only one method (disk zone diameter on YNB agar) demonstrated a correlation, but this correlation could not be used to establish a MIC breakpoint because of the relatively narrow range of ED_{50} values seen in the animal model as well as difficulties in reading the zone diameters.

Second, the in vitro test must demonstrate differences between isolates in order to be predictive. In the study by Fisher et al. (47), only three *Candida* isolates were tested. For all three the fluconazole MICs were >100 μg/ml. Fluconazole was active in vivo against two of the three strains, but without differences in MICs it is not possible to discern a correlation. It is not sufficient to assume that a numerically large MIC implies resistance: the above review of technical variables clearly demonstrates that the test conditions can be manipulated to produce MICs in any desired range.

Third, interpretation of the results must take drug pharmacology into account. Rogers and Galgiani (127) studied two *C. albicans* isolates: the MICs of both ketoconazole and fluconazole were low for one isolate and high for the other. Fluconazole was effective therapy for the isolate with the lower MIC but not for the isolate with the higher MIC. On the other hand, despite having a 16-fold lower MIC than fluconazole against the susceptible isolate, ketoconazole was ineffective in this intravenous infection model. This discrepancy could be due to the greater protein binding and shorter half-life of ketoconazole. Moreover, since the kidney is the primary target organ in this experimental model, the fact that fluconazole is concentrated and excreted in the urine while ketoconazole is not may also be important.

In another demonstration of the relevance of host factors, Boyle et al. (14) studied a large number of variously substituted azole derivatives both in vivo and in vitro. They found that, when grouped by class of substitution, those classes of molecules that would be expected to be relatively resistant

TABLE 2. Relationship between in vitro susceptibility test results and in vivo efficacy of antifungal agents in animal models

Drug (reference)	Model(s) and fungus (no. of strains)	In vitro method, medium, inoculum (CFU/ml), temp (0°C), time	In vitro-in vivo correlation
Amphotericin B (3)	Mouse/i.v.,[a] *C. albicans* (9)	Microbroth, other details not stated	Yes
Amphotericin B (47)	Mouse/i.v., *C. tropicalis*, *T. glabrata*, and *C. krusei* (1 each)	Macrobroth, antibiotic medium 3, 10^6/ml, temp not stated, 48 h	Not clearly
Amphotericin B (34)	Mouse/i.v., *C. lusitaniae* (4)	Microbroth, RPMI 1640, 10^4/ml, 35°C, 24 h	Yes
Amphotericin B and amphotericin B methylester (104)	Rabbit meningitis, *C. neoformans* (1); and rabbit endocarditis and pyelonephritis, *C. albicans* (1)	Macrobroth, Sabouraud agar, 10^3–10^4/ml, 24 h, 30°C	No
Amphotericin B and natamycin (93)	Rabbit keratitis, *C. albicans* (17)	Microbroth, antibiotic medium 3, 10^5/ml, 24 h, 37°C; also by an agar dilution method	Yes for microbroth MIC; microbroth MFC[b] and agar dilution methods not as good
5-Fluorocytosine (3)	Mouse/i.v., *C. albicans* (9)	Microbroth, other details not stated	Yes
5-Fluorocytosine (34)	Mouse/i.v., *C. lusitaniae* (4)	Microbroth, RPMI 1640, 10^4/ml, 35°C, 24 h	Yes
5-Fluorocytosine (122)	Mouse/i.v., *C. albicans* and *C. tropicalis* (1 each)	Microbroth, RPMI 1640, 10^3/ml, 24 h, 35°C	Yes
5-Fluorocytosine (139)	Mouse/i.v., *C. albicans* (40)	Macrobroth, YNB, 10^3/ml, 7 days, 37°C	Yes
Ketoconazole (122)	Mouse/i.v., *C. albicans* and *C. tropicalis* (1 each)	Microbroth, RPMI 1640, 10^3/ml, 24 h, 35°C	Yes
Ketoconazole (128)	Mouse vaginosis, rat vaginosis, and mouse/i.v., *C. albicans* (43)	Various agar and broth methods	Yes for all but one agar dilution method
Ketoconazole (112)	Mouse/i.v., *C. albicans* (58)	16 different agar and broth methods	Slight correlation with one method; none with others
Ketoconazole and fluconazole (72)	Rabbit/subcutaneous chamber, *C. albicans* (5)	Macrobroth, YNB, 10^5/ml, 48 h, 37°C	Yes
Fluconazole (34)	Mouse/i.v., *C. lusitaniae* (4)	Microbroth, RPMI 1640, 10^4/ml, 35°C, 24 h	Yes
Fluconazole (47)	Mouse/i.v., *C. tropicalis*, *T. glabrata*, and *C. krusei* (1 each)	Macrobroth, SAAMF, 10^6/ml, 48 h, temp not stated	Not clearly
Fluconazole (127)	Rat/i.v., *C. albicans* (2)	Macrobroth, buffered SAAMF, 10^3/ml, 22 h, 37°C	Yes
Fluconazole (148)	Mouse/intracerebral, *Cryptococcus neoformans* (20)	Macrobroth, SAAMF, 0.5×10^3–2.5×10^3/ml, 24 and 48 h	Yes at 24 h but no at 48 h
Fluconazole and itraconazole (105)	Rabbit pyelonephritis, *C. albicans* (2)	Macrobroth, SAAMF, 10^5/ml, 24 h, 30°C	Yes
SCH39304 (85)	Rat/i.v., *C. albicans* (2)	Macrobroth, buffered SAAMF, 10^3/ml, 22 h, 37°C	Yes
Bay N 7133 (109)	Mouse/i.v., *C. albicans* (59)	Agar dilution, Isotonic broth, 10^4/ml, 48 h, 28°C	No
Multiple mono- and bis-triazoles (14)	Mouse vaginosis, *C. albicans* (1)	Macrobroth, YNB, 10^5/ml, 48 h, 37°C	Yes
Cilofungin (86)	Rat/i.v., *C. albicans* (2)	Macrobroth, buffered SAAMF, 10^3/ml, 22 h, 37°C	No

[a] i.v., intravenous.
[b] MFC, minimal fungicidal concentration.

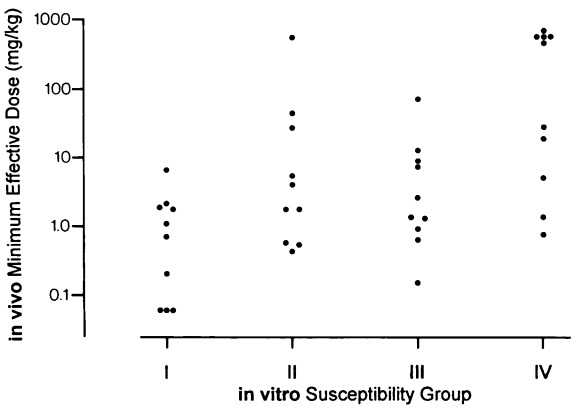

FIG. 2. Correlation of 5-fluorocytosine MIC with minimum effective dose of 5-fluorocytosine. Responses to 5-fluorocytosine therapy in mice infected with four different groups of *C. albicans* are shown. Susceptibility group I contains the most susceptible isolates, while susceptibility group IV contains the most resistant isolates. Data are from Stiller et al. (139) and are reproduced with permission of the publisher.

to metabolic degradation by the host had a good in vitro-in vivo correlation whereas compounds that were predicted to be readily degraded did not demonstrate a good correlation.

Only one study has sought an in vivo-in vitro correlation for cilofungin (86). This study was discussed above, and there was no correlation despite demonstration of a tantalizing range of cilofungin MICs at one pH but not another.

Clinical Demonstrations of Both Inherent and Acquired Resistance

Polyene resistance has been said to be rare, but its exact incidence is hard to determine. A number of studies have demonstrated rises in amphotericin B MICs for isolates obtained during prolonged polyene therapy, and these rises have sometimes appeared to correlate with clinical failure (13, 33, 38, 46, 103). In one oft-cited study (32), isolates from 6 of 70 cancer patients exhibited amphotericin B resistance (defined as a MIC of ≥ 2 μg/ml) by an agar dilution technique. Five of these six patients had received extensive polyene therapy, but additional clinical details were not provided. In a later report, Powderly et al. (120) examined bloodstream isolates from patients undergoing bone marrow transplantation. Using a macrobroth technique in yeast extract broth at 30°C with 10^6 yeast cells per ml and read at 48 h, they found a higher mean amphotericin B MIC for the bloodstream isolates than for the control isolates from nonpatients, even though many of these patients had not received amphotericin B previously. Further, they found that isolates for which the MIC was >0.8 μg/ml more often caused fatal infections. While this study is limited by its retrospective nature and difficulties in classifying outcome, a similar retrospective study also found a higher crude mortality among patients with bloodstream infection due to *Candida* isolates with an amphotericin B MIC of >0.78 μg/ml (17). Finally, the isolates from an AIDS patient with cryptococcal meningitis who relapsed while on maintenance fluconazole (119) and then failed to respond to amphotericin B were studied for amphotericin B susceptibility by a macrobroth technique in yeast extract at 30°C with 5×10^5 yeast cells per ml and read at 24, 48, and 72 h. By comparison with the pretreatment isolate, the amphotericin B MIC for the posttreatment isolate was found to have risen fourfold but to also be less virulent in a mouse model. These correlations are not overwhelming, nor have they been consistently observed by others. Hughes et al. (73) recently tested 60 *Candida* bloodstream isolates for amphotericin B susceptibility by a broth method in RPMI 1640 with 5×10^4 yeast cells per ml at 37°C for 48 h. While the MICs for all isolates were 0.5 to 2.0 μg/ml, those for strains of *C. parapsilosis* were 1.0 and 2.0 μg/ml and MICs for strains of *C. albicans* were 0.5 or 1.0 μg/ml. Despite this trend towards lower susceptibility of the *C. parapsilosis* strains, the patients infected with *C. parapsilosis* had a higher frequency of resolution of infection. It is difficult to interpret these conflicting data on polyene resistance. While amphotericin B MICs, by at least some techniques, do seem to rise following therapy (9, 32, 119), the more resistant organisms may be less virulent (9, 119), and it is often difficult to truly ascribe failure to the drug in the face of the multiple immune system defects present in the typical patient.

Although excellent animal model data are available (Table 2), 5-fluorocytosine is now rarely used alone to treat patients, and well-studied cases describing the correlation between in vitro and in vivo results are not available. In an unpublished doctoral thesis (28), Deldicque described a small number of cases in which 5-fluorocytosine susceptibility results appeared to correlate with outcome of 5-fluorocytosine monotherapy. Unfortunately, clinical details are limited and it is hard to draw a firm conclusion.

Azole resistance has often been described. In an early report, initial clinical response was followed by relapse during prolonged use of oral miconazole: the sequential isolates demonstrated a 200- to 500-fold rise in MIC by an unspecified method (69). In another set of reports, three isolates from patients who relapsed after prolonged continuous ketoconazole therapy for chronic mucocutaneous candidiasis (70, 136) and one from a patient who never responded (151) were described and studied. These *C. albicans* isolates are clearly more resistant to ketoconazole by tests of inhibition of hyphal elongation (151) and by both agar dilution and macrobroth techniques (75, 105, 127, 128). Three of the four isolates were studied in more detail and found to be cross resistant to miconazole, ketoconazole, itraconazole, and fluconazole by agar dilution, macrobroth, inhibition of germ tube elongation, and relative inhibition factor techniques (136). Two of these isolates were also studied in animal models and found to be probably more resistant to therapy in several different animal models (136) and definitely more resistant in a rabbit pyelonephritis model (105). It is interesting to note that in both of these studies a larger inoculum of the resistant isolate was required to produce infection, implying a lowered virulence, and it is this difference in virulence that makes the animal data from the first study (136) hard to interpret. This finding of apparently reduced virulence is not universal, however. When Rogers et al. (127) and McIntyre and Galgiani (85) tested one of the above-described resistant isolates in a rat model of fluconazole or SCH39304 therapy, respectively, the required inoculum was identical to that needed for the susceptible strain of *C. albicans*.

Radetsky et al. (122) collected 84 *Candida* isolates and determined drug MICs in a microbroth system with a variety of media and antifungal agents, a starting inoculum of 10^3 per ml, and incubation at 35°C for 24 h. Use of RPMI 1640 was found to produce a broad spectrum of MICs. The authors then present a small amount of data that correlates clinical outcome with MIC results (using RPMI 1640) obtained for

the isolates from seven patients who had been treated with a single antifungal agent (ketoconazole, 5-fluorocytosine, or amphotericin B). While a correlation appears to exist, insufficient data are presented to support a firm conclusion.

Because of its ready oral and intravenous administration, fluconazole is widely used for therapy of candidiasis and cryptococcosis. As with ketoconazole, reports of clinical resistance have begun to appear, and studies of some of these isolates have demonstrated relative in vitro resistance. Willocks et al. (152) described four AIDS patients with oropharyngeal candidiasis who failed fluconazole therapy. The MICs for these patients' isolates as determined by an unspecified broth method were four- to eightfold higher than those for a group of control isolates. Using the proposed NCCLS reference methodology, Rodriguez-Tudela et al. (126) have briefly reported on strains from six AIDS patients with oropharyngeal candidiasis who failed fluconazole therapy, and MICs for these isolates were ≥16 μg/ml. Finally, elevated MICs for isolates from a group of AIDS patients with persistent oropharyngeal candidiasis despite fluconazole therapy were found by a microtiter method, using HR media (other details unspecified), and these isolates were also more virulent in an undescribed mouse model (39).

Of particular interest are the multiple reports of both prophylactic and therapeutic failures of fluconazole against *C. krusei* (1, 2, 10, 23, 84, 106, 125, 153). The largest of these studies is a retrospective study of 463 bone marrow transplant or leukemia patients (153). There was a sevenfold-greater (8.3 versus 1.2%) incidence of bloodstream or visceral infection with *C. krusei* in the 84 patients who received fluconazole prophylaxis in comparison to the 355 patients who received other forms of prophylaxis, including ketoconazole, miconazole, and amphotericin B, or no prophylaxis. This finding has been reported in some (106, 141), but not all other, comparable studies (22, 58). Taken together, these data strongly suggest that while there may be local epidemiologic factors that affect the incidence of *C. krusei* infection (131), *C. krusei* is often resistant to fluconazole. While MICs for individual *C. krusei* isolates have been reported as elevated by some authors, only one report directly comparing the MICs for *C. krusei* with those for other *Candida* spp. is available (81). It demonstrated higher MICs for *C. krusei* than for other species. Comparable data are not yet available for the proposed NCCLS reference method.

Clinical Trials

In only a few therapy trials have isolates been collected and tested and data correlated with clinical outcome, and none provide useful results. In a trial of 400 versus 800 mg of ketoconazole per day for progressive forms of coccidioidomycosis, the isolates were tested by a broth dilution method (55, 74). The MICs for all of the isolates were similar, and no correlation with therapeutic outcome could be drawn. In a second study, 69 isolates from 50 of 52 patients enrolled in a collaborative trial of ketoconazole as therapy for blastomycosis ($n = 21$), coccidioidomycosis ($n = 24$), histoplasmosis ($n = 9$), nonmeningeal cryptococcosis ($n = 7$), and sporotrichosis ($n = 8$) were tested by a macrobroth system with a variety of media, an inoculum of 2.5×10^4 per ml, and incubation at 30°C for 48 to 96 h (134). The in vitro results were not predictive of clinical outcome, even when isolates obtained from patients who had relapsed during therapy were tested. Finally, in a retrospective review of cases of sporotrichosis treated with ketoconazole, the range of MICs was quite narrow and not predictive of outcome (20).

SUMMARY

The prospect for meaningful, reproducible antifungal susceptibility testing, at least of *Candida* spp. and *Cryptococcus neoformans*, appears bright. It is clear from a wide variety of data that a correlation between in vitro susceptibility testing and in vivo efficacy is likely to exist when the proper test conditions are selected. Although the absolute MICs obtained vary with technique, the rank order of the isolates appears to be constant and very telling (18, 19, 51, 108, 152). Thus, no matter what method is used, its range of MICs will need to be correlated with animal model data or clinical outcomes: it is simply not possible to try to predict the meaning of a MIC on the basis of whether it is higher or lower than achievable serum levels of that drug. To facilitate cooperative testing and to allow analysis of clinical isolates, a reproducible reference method is at hand. While cumbersome, it will provide a touchstone during cooperative studies to define improved techniques. Several other major areas for immediate work are apparent. First, large numbers of isolates from well-designed clinical therapy trials need to be tested by the reference method, and the results need to be correlated with clinical outcome. Such studies are already under way, and the results should be available in the near future. As there is no guarantee that results obtained by the proposed reference method will be predictive of clinical outcome, variant procedures should also be examined. By designing variations that follow the spirit of the proposed reference method, investigators are reasonably assured that a new method could be reproduced in other laboratories. Second, it appears that the reference method may not be sensitive to amphotericin B resistance, and careful work will be needed to resolve this issue. Third, the relevance of MLCs rather than MICs will need to be evaluated, and there will likely be many technical issues that must be resolved before a reproducible method for MLC determination is available. Fourth, increasingly detailed studies of the mechanisms of antifungal resistance of selected isolates have become available (65, 76, 77, 89, 147), and such isolates should prove to be useful for further validating any MIC methodology. Finally, all of these studies may benefit from the application of molecular genetic analysis. For example, Casadevall et al. studied sequential isolates from five patients with recurrent cryptococcal meningitis treated with fluconazole (21). These authors found that the isolates were clonally related and all had the same MIC by the proposed NCCLS reference method, leading the authors to conclude that the clinical failures in these patients were because of changes in immune function or lack of compliance rather than due to failure of fluconazole therapy.

Susceptibility testing of the filamentous fungi is currently poorly developed. Standardized methods for testing of asexual conidia could probably be extrapolated from those used for yeasts, but it may not be clinically relevant to test this phase of the organism. Tests of antifungal susceptibility of hyphae need to be developed. While such tests are likely to be technically challenging, correlation of these results with testing of conidia and other asexual propagules and clinical outcome could then take place, allowing an appropriate testing procedure to be defined.

Implications for the Clinical Laboratory

While promising, antifungal susceptibility testing is still a research tool. It should not be used routinely since, in the absence of local experience with a given assay and its relevance to clinical outcome, it is not generally possible to interpret the results. The information needed to determine MIC breakpoints for the proposed NCCLS method is not available, nor is it reasonable to arbitrarily select MIC breakpoints on the basis of achievable serum concentrations of an antifungal agent. Although testing of serial isolates during long-term treatment might reveal a rising MIC and thus suggest resistance, clinical response rather than susceptibility testing results remains the most reliable guide to therapy at the present time.

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